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TITLE:

Inter-sample contamination detection using mixture deconvolution comparison

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HIGHLIGHTS:

- We trial a mixture to mixture comparison tool in a quality assurance context
- All samples within eight extraction batches and four run batches were compared
- Two mechanisms of potential sample to sample contamination were investigated
- No instances of sample to sample contamination were identified
- The potential for sample to sample intelligence gathering was explored

ABSTRACT:

A recent publication has provided the ability to compare two mixed DNA profiles and consider their probability of occurrence if they do, compared to if they do not, have a common contributor. This ability has applications to both quality assurance (to test for sample to sample contamination) and for intelligence gathering purposes (did the same unknown offender donate DNA to multiple samples). We use a mixture to mixture comparison tool to investigate the prevalence of sample to sample contamination that could occur from two laboratory mechanisms, one during DNA extraction and one during electrophoresis. By carrying out pairwise comparisons of all samples (deconvoluted using probabilistic genotyping software

STRmix™) within extraction or run batches we identify any potential common DNA donors and investigate these with respect to their risk of contamination from the two proposed mechanisms. While not identifying any contamination, we inadvertently find a potential intelligence link between samples, showing the use of a mixture to mixture comparison tool for investigative purposes.

KEYWORDS:
Contamination; extraction batch; deconvolution; mixture comparison.

1.0: INTRODUCTION:

It is standard practice in a forensic laboratory to have a system of quality assurance (QA) that involves a number of quality control (QC) checks at various points of an analytical or reporting workflow. Within forensic biology this typically includes checks for contamination, where the two most common forms of QC are:

1) Checks for contamination occurring in reagents – facilitated by the inclusion of reagent blanks associated with either the extraction or the PCR set-up.
2) Checks for person to sample contamination – facilitated by comparison of the generated evidence profiles with a register of people who may have inadvertently contaminated the sample during its collection or processing in the lab.

The advent of probabilistic genotyping (PG) systems has led to more, and more complex, DNA profiles being evaluated as part of routine casework [1] (for example, validation studies of PG systems now routinely include complex DNA profiles from five individuals [2, 3]). Analysing profiles of this complexity, requires an equivalently sophisticated ability to carry out QC checks for contamination. The ability to carry out mass screening of a database of individuals against a complex profile deconvolution [4] provides this ability and its use as a QA tool to this effect has been demonstrated for laboratory environmental swabbing [5].

These advances in contamination checking still require that one of the samples being compared originates from a single source. The database screening process then allows a likelihood ratio (*LR*) to be calculated to each person of interest (POI) on the database using propositions, which are typically in the form:

- The sources of DNA are the POI and $N - 1$ unknown individuals
- The sources of DNA are N unknown individuals

for an N person mixture.

Being limited to profile comparisons where one of the profiles must be single sourced drastically limits the ability to carry out sample to sample contamination checks (as many evidence samples originate from more than one contributor with unresolvable components).

As a result, sample to sample contamination checks are not routinely carried out as part of a normal QA process.

Recently Slooten [6] outlined a method whereby an *LR* could be calculated, comparing two DNA profiles, without the restriction that one must be single sourced. This *LR* uses propositions for each pairwise comparison of mixture components. When comparing two mixtures M and M', we evaluate the following propositions:

$H_{1,i,j}$: $D_i = D_j'$ and all other donors of the mixtures are unrelated,

H_2: all donors of both mixtures are unrelated,

where donors *i* and *j* are defined with respect to mixtures M and M' respectively. If one of the two samples being compared happens to be single source, then the propositions can be restated in the usual manner as given in the earlier proposition pair. This opens the possibility to carry out routine sample to sample contamination checks that utilise the deconvolution carried out by a PG software.

A software tool has been developed that utilises the mathematics of Slooten [6] and applies it to deconvolutions carried out by STRmix™ [7-9] (https://www.strmix.com). This tool has been shown to have a high performance when comparing mixtures that either have no, or some, contributors in common [10]. In this paper we carry out experiments using this feature within a QA context. We concentrate on two types of potential QA issues that have arisen at Forensic Science SA (described in section 2) and evaluate their impact. In both instances the mechanisms (which are described in section 2) are likely to only lead to low level contamination (if it occurs at all) and so the identification of contamination is realistically only identifiable with the use of PG systems.

2.0: DESCRIPTION OF POTENTIAL SAMPLE TO SAMPLE CONTAMINATION EVENTS:

2.1: Sample to sample contamination during DNA extraction

The first potential contamination event is one that could have occurred during the DNA extraction stage. For the samples included in our study, DNA extraction was carried out using a Perkin-Elmer MP2 liquid handling platform. The DNA extraction (shown diagrammatically in Figure 1) involved several steps where DNA samples were mixed using pipetting solution back and forth. Between mixing steps, the tips were returned to their original holder position in a deep welled holding plate. After mixing steps the tips were discarded. Because each tip was only ever used to mix one sample, the reuse of the tips was not thought to introduce any contamination risks to the process. However, after some extractions three risk factors were identified:

1) The deep well tip holder only divided the plate into blocks of four tips, and not into individual tips (as shown in Figure 1),
2) It was noticed during one extraction that some micro-droplets of DNA extract were present on the tips as they were returned to the deep well tip holder,
3) On a particular DNA extraction batch, a very weak contamination event of a reagent blank on the extraction plate could be explained by a sample (that had an extremely high DNA load) in one of the deep well quadrants.

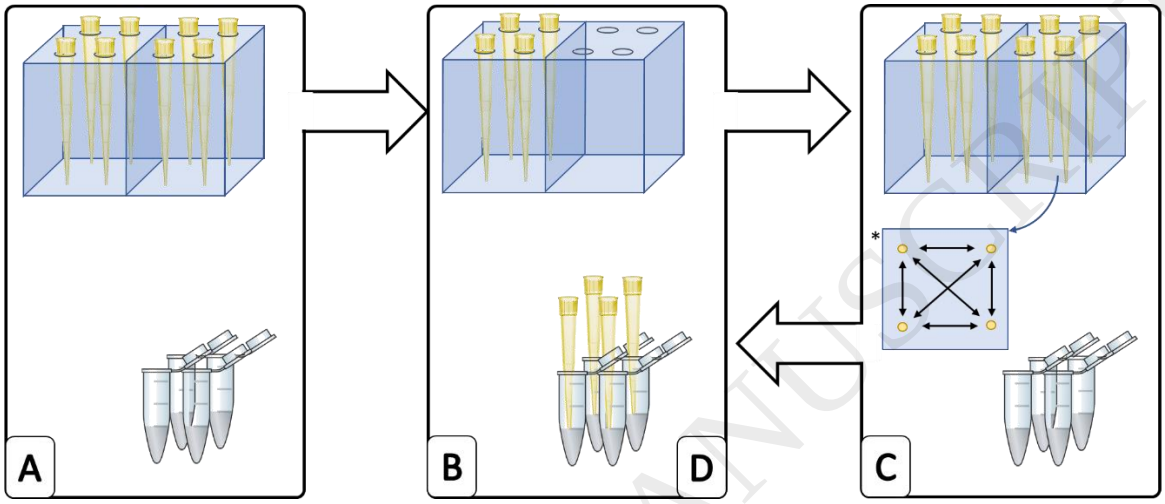


Figure 1: Diagrammatic representation of four steps in an automated DNA extraction process. Step A shows clean tips in their holders and four DNA samples awaiting DNA extraction. Step B shows the use of four of the tips to carry out part of the extraction. Step C shows the used tips being returned to their holder and the asterisk (*) inset shows the potential pathways for DNA extract droplets to be transferred from tip to tip. Step D shows the re-use of the tips on the DNA extracts (in the same configuration as in step B), but now with the potential for contamination to have occurred.

It was surmised that the combination of micro-droplets on the used tips, deep-well holders breaking up the tips into lots of four tips, and the re-use of tips for DNA extract mixing could be vectors for introducing sample to sample contamination at the DNA extraction stage.

As a result of this potential contamination the DNA extraction procedure was altered so that (in one part of the change) tips were discarded after every use.

2.2 Sample to sample contamination during electrophoresis

The second potential contamination event is one that could occur during electrophoresis. For the samples used in our study, electrophoresis was conducted using an ABI 3500xl capillary electrophoresis instrument. The analysis of multiple samples (shown diagrammatically in

Figure 2) occurs in a block of 'injections'. Each injection electro-kinetically loads samples on to the capillary in a block of 24 for the 3500xl or 16 for the 3130xl (note, diagrammatically, the injection size shown in Figure 2 is four). Each capillary is cleaned automatically with buffer between injections in order to minimise (and ideally completely remove) any PCR products remaining from the previous injection. The contamination of one well, via a capillary, with PCR products from the well that the capillary previously injected is called capillary carry-over and anecdotally has been observed when one sample is heavily loaded with PCR products.

At Forensic Science SA an instance of a PCR positive control sample showing the presence of a weak, second contributor has previously been obtained. The minor contributor could be explained by the DNA in the previously injected well for that capillary.

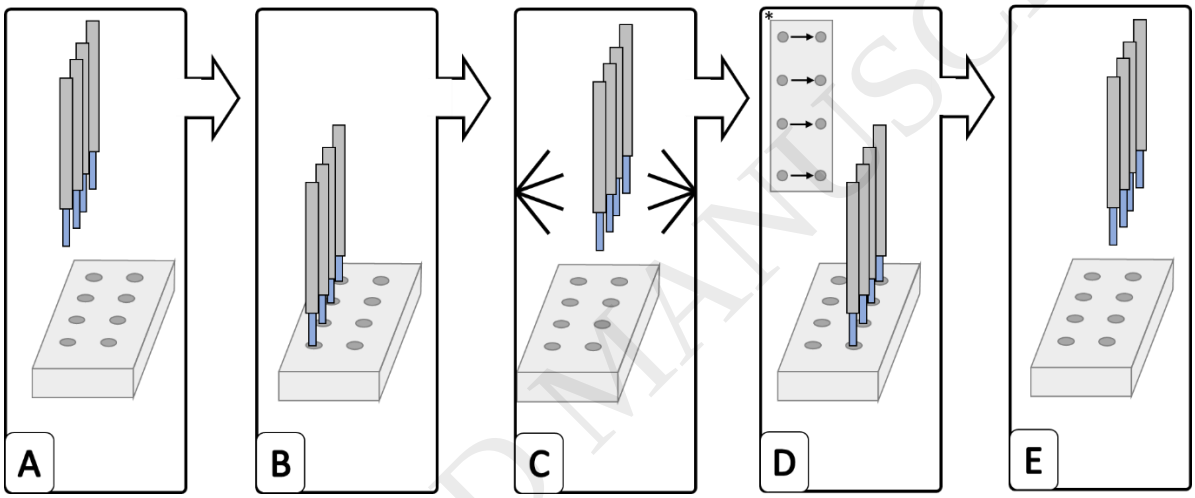


Figure 2: Diagrammatic representation of five steps in an electrophoresis run. Step A shows clean capillaries prior to any sample loading. Step B shows the immersion of tips in the first row of samples being loaded. Step C shows the capillaries between injections going through a cleaning cycle. Step D shows the second injection of the instrument, and the asterisk (*) inset shows the potential pathways for PCR products to be transferred from capillary to capillary. Step E shows the capillaries after the sample injections.

3.0: METHODS:

3.1: Sample analysis

All samples chosen had been profiled using GlobalFiler™, amplified on a GeneAmp® 9700 or ProFlex™ thermocycler (ThermoFisher Scientific) and separated on either an Applied Biosystems 3130*xl* or 3500*xl* Genetic Analyzer (ThermoFisher Scientific). Electropherograms (EPGs) were read using GeneMapper® ID-X v1.4 with an analytical threshold of either 30 rfu (for data run on 3130*xl*) or 50 rfu (for data run on the 3500*xl*). Specific STRmix™ settings used to analyse samples were obtained from in-house laboratory validation and, as samples

chosen for this study spanned a number of version changes, there were various STRmix™ settings in use.

To address sample to sample contamination during DNA extraction we examined four extraction batches leading up to the change in extraction protocol and four extraction batches after the change. Only samples that had been deconvoluted as part of casework were included in the study (i.e. when samples were deemed to be too complex for analysis or a number of contributors could not be determined, then deconvolution was not attempted by the original case-working scientist and not subsequently attempted for our study). Summary values for the eight extraction batches are given in Table 1.

To address the potential contamination at the electrophoresis stage we examined four electrophoresis 'run batches'. As before, only samples that had been deconvoluted as part of casework were used in the analysis and all intra-case comparisons were screened out prior to comparison. Summary values for the four run batches are given in Table 2.

Intra-case comparisons were screened out as there is a legitimate reason why there may be common contributors to these sample in these cases, and coupled with the unknown specifics surrounding the samples, they are of limited value for our purposes. When carrying out inter-case comparisons the assumption is that it is much less likely to have legitimate common contributors to samples.

3.2: *LR* calculation

As per the method outlined in [10], for each mixture to mixture comparison, an *LR* stratified over the contributors was computed, i.e. an *LR* was computed for the propositions:

H_1: the two mixtures share one common donor,

H_2: all donors of both mixtures are unrelated,

where the *LR* is the average of all corresponding pairwise *LR*s.

$$LR = \frac{1}{NN'} \sum_{i=1,\ldots,N} \sum_{j=1,\ldots,N'} LR_{i,j}$$

where the mixtures have N and N' contributors. All *LR* calculations are carried out using the Australian Caucasian allele frequency data [11] and make no corrections for sub-population stratification. $\text{Log}_{10}(LR)$ values were bounded between -10 and +10 for visual clarity, with any values falling outside these ranges given the appropriate bounding value.

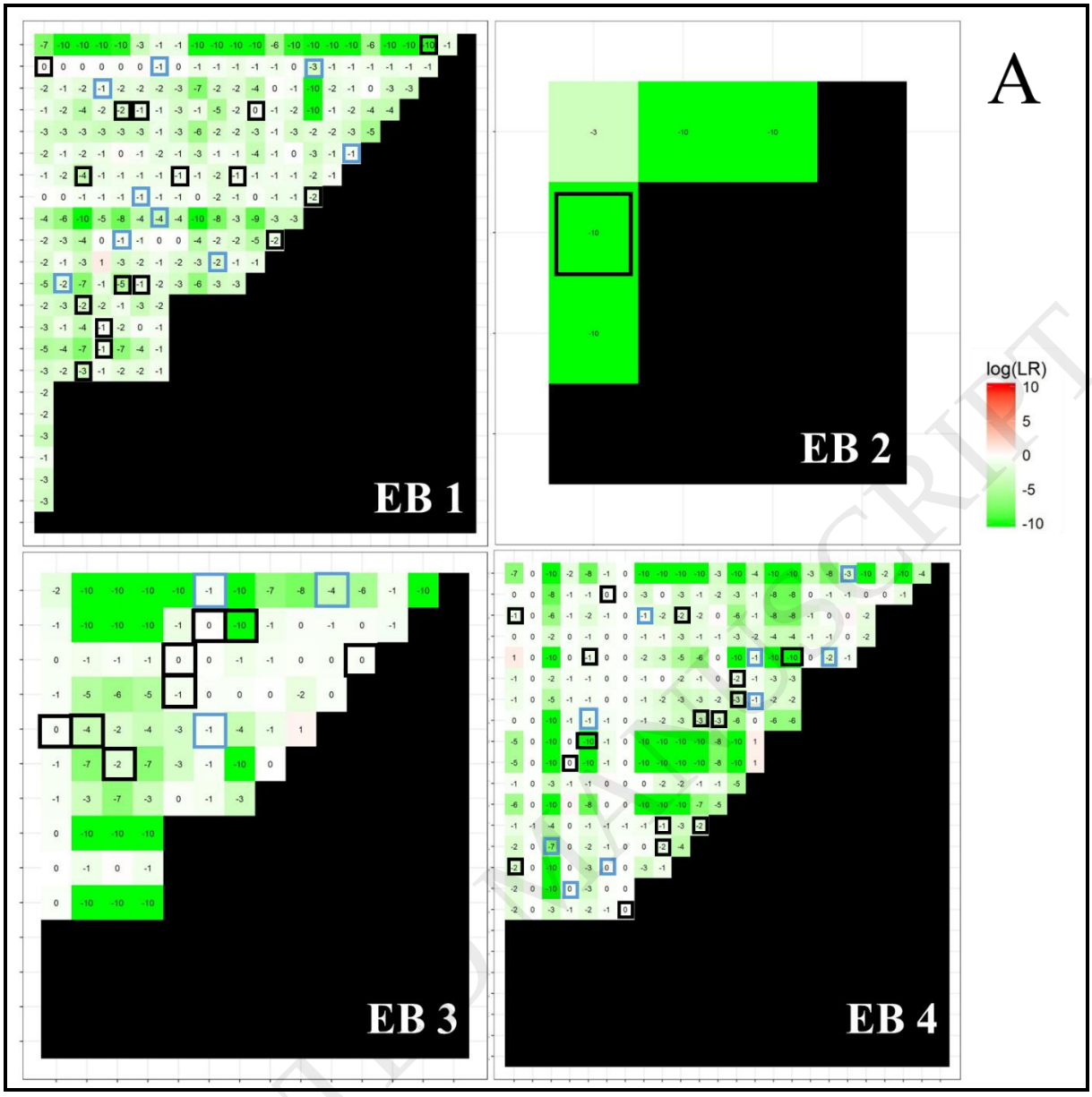
4.0: RESULTS:

Analyses were run on a PC with an Intel Xeon E3-1505M v5 CPU operating at 2.8GHz, and 64GM RAM running Windows 10. The algorithm to carry out mixture comparisons was first programmed in C# and has now also been implemented in Java in STRmix™ V2.7. Running time depends on the number of comparisons to be carried out and the complexity of the mixtures within the batch, however as an indication of the general timeframes, a full pair-wise comparison of 340 samples (57,000 comparisons), ranging from 1 to 5 person mixtures took 85 minutes to complete on the described system. The number of pairwise comparisons grows quadratically with the number of samples and so does the runtime.

4.1: Inter-sample contamination findings

Figure 3 shows the distribution of $\log_{10}(LR)$ values for the sample to sample comparisons within extraction batches before (Figure 3A) and after (Figure 3B) the change in DNA extraction methodology involving tip re-use. In Figure 3, each sample within each extraction batch is listed on both axes and the cells represent the $\log_{10}(LR)$ comparison of the samples corresponding to the column and row of the plot. The cells are coloured from green, corresponding to $\log_{10}(LR) = -10$, (and hence extremely strong support for no common contributors) to red, corresponding to $\log_{10}(LR) = 10$, (and hence extremely strong support for a common contributor).

The lower right diagonal of each plot in Figure 3 is black as this represents the complementary comparison (i.e. comparing B to A, when we have already given the results of the comparison of A to B). Additionally, intra-case sample comparisons are blacked out (causing some cells near the diagonal to be black).



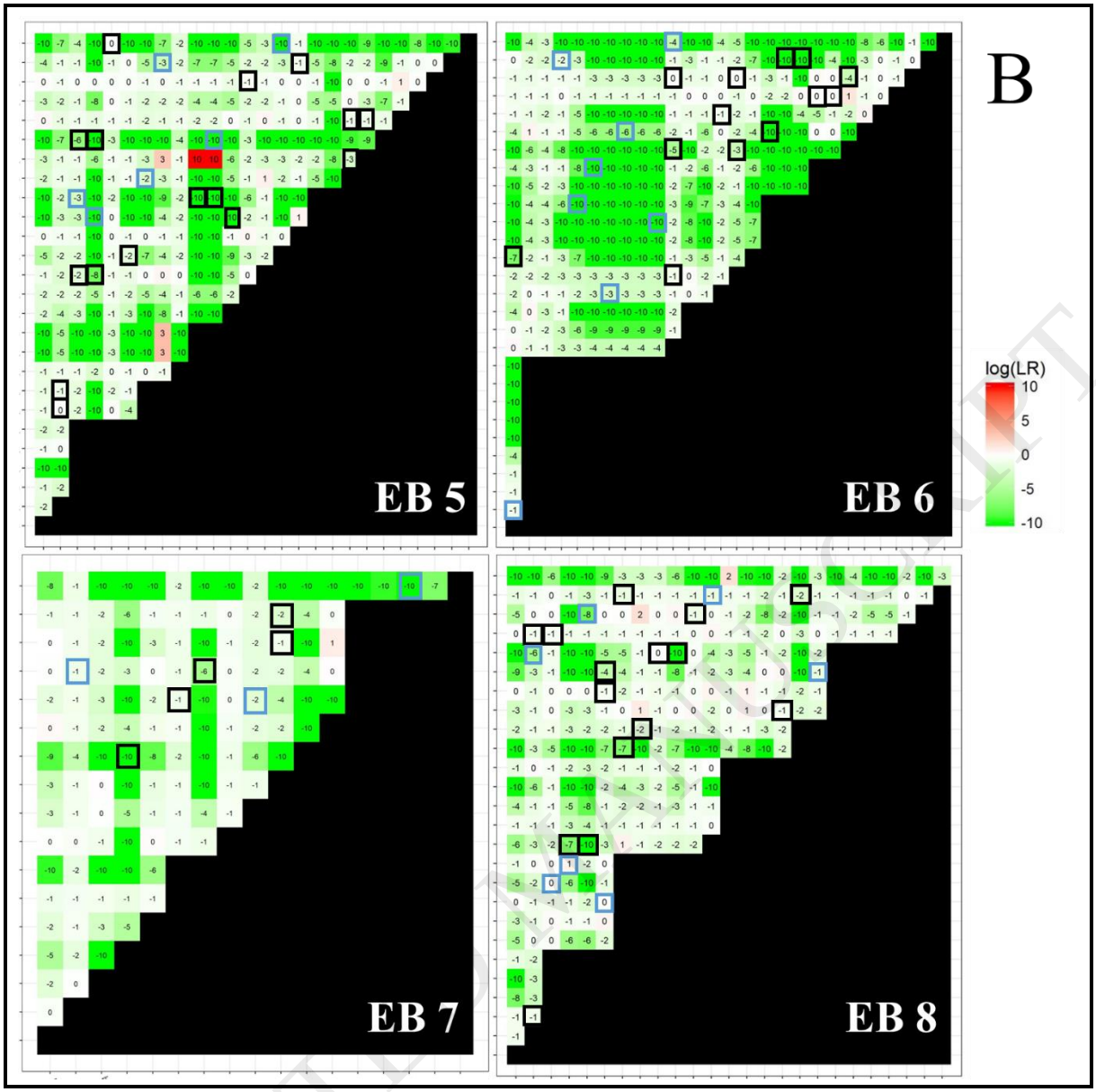


Figure 3: Log_{10}(LR) for sample to sample comparisons for samples extracted before (A, top) and after (B, bottom) extraction methodology change. Cells represent the comparison between samples listed in the column and row of the table. Log_{10}(LR) values are represented by colour (as per legend) and by numerical value in each cell. Black bordered cells in section represent those that fall into the potential extraction batch contamination. Blue bordered cells are those that fall into the potential electrophoresis batch contamination.

Figure 4 shows the comparison of samples within run batches. As with Figure 3, the results are of comparisons between all samples within the batch. Samples that fall into the potential capillary carry-over contamination window indicated by a blue border and those that fall into the potential extraction batch contamination window are indicated by a black border. Even though the purpose of the comparisons in Figure 3 are contamination at the extraction stage and in Figure 4, contamination at the electrophoresis stage, borders indicating potential contamination for both types of contamination are indicated on both Figures. The reason for

this is that to visualise the DNA profiles (and hence carry out analysis using STRmix) the samples must have undergone an extraction and electrophoresis, and due to laboratory work-flow, it is common that samples within an extraction batch will also be on the same run batch.

Also note that while the re-use of tips during DNA extraction was changed as a result of the potential for contamination to occur between samples which had tips in the same 'quadrant' (extraction batches shown in Figure 3A), we still show the equivalent samples bordered by black boxes in Figure 3B so that a direct comparison of the relevant samples can be carried out.

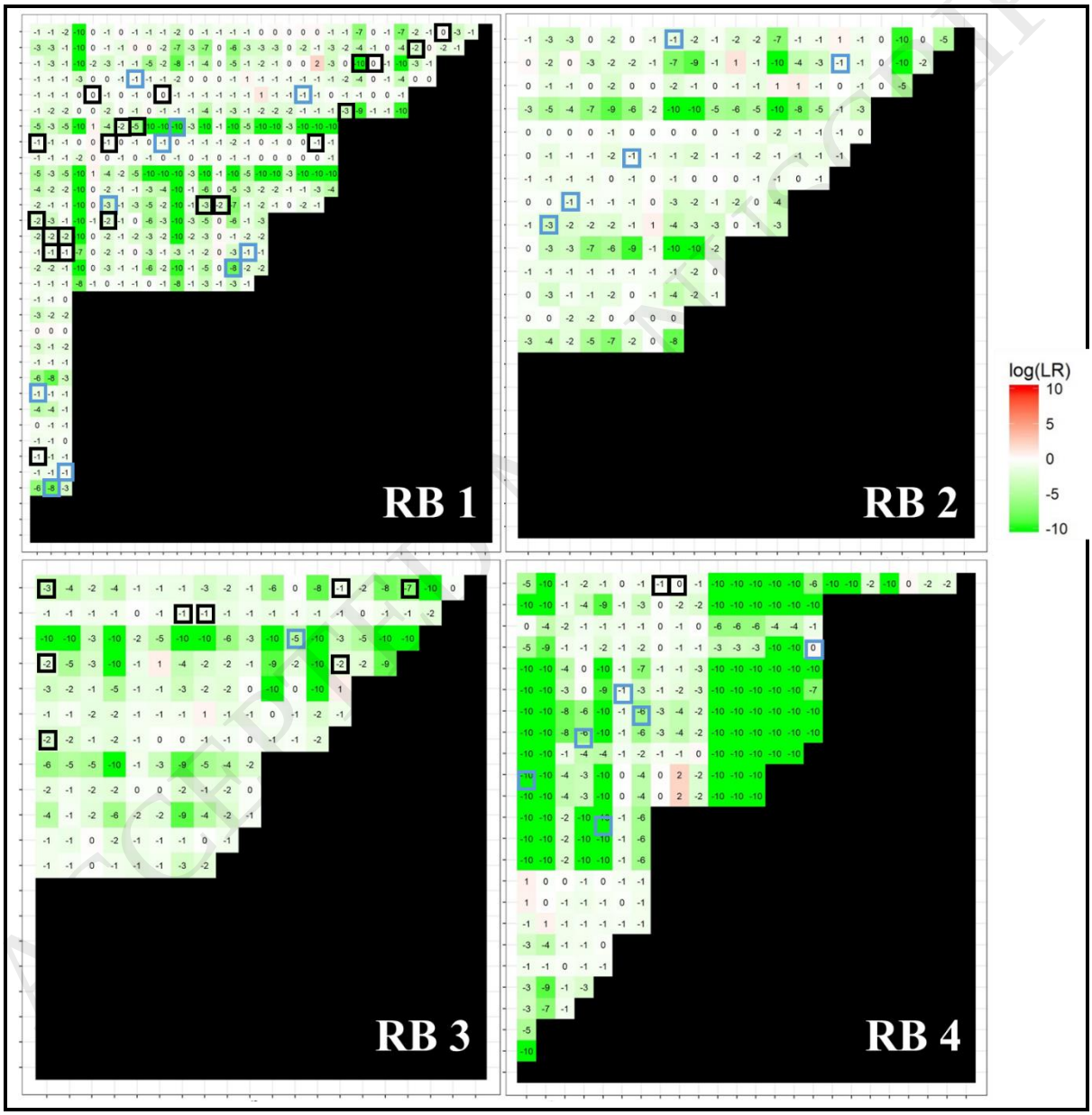


Figure 4: Log_{10}(LR) for sample to sample comparisons for samples within run batches. Cells represent the comparison between samples listed in the column and row of the table. Log_{10}(LR) values are represented by colour (as per legend) and by numerical value in each cell. Black bordered cells in section A represent those that fall into the potential extraction batch

contamination. Blue bordered cells are those that fall into the potential electrophoresis batch contamination.

5.0: DISCUSSION:

5.1: Investigation into putative inter-case contaminations

As can be seen in Figures 3 and 4 there was a range of *LR* values obtained for mixture to mixture comparisons. As expected for the majority of these samples the analysis supported the proposition that there was not a common contributor to the samples, and hence (in the QA context that we tested them in) that contamination had not occurred. The point at which a laboratory considers the level of support for any *LR* based contamination screen is somewhat arbitrary and usually has a component of resource availability within the decision-making process. For our study we choose an *LR* threshold of investigation of 1 million (which aligns with the mixture searching threshold in use at Forensic Science SA). In both mechanisms of potential contamination, the level of sample to sample contamination (if it did occur) is likely to be low. Under this criterion there was only two instances of potential contamination within extraction batch 5 (seen as two dark red squares with $\log_{10}(LR) = 10$ in Figure 3B). However, these comparisons did not fall into the window expected from the two potential contamination mechanisms investigated in this paper. Further investigation into these samples found that the two sample to sample comparisons arose from one sample in one case and two samples from a second case, and that both cases had the same individual nominated as the suspect. If indeed the nominated individual was involved in both matters, this would account for the *LR* obtained from the sample to sample comparison, without the need to invoke a contamination event. While not a main focus of the study, this finding demonstrates a second use for mixture to mixture comparison (other than for quality assurance purposes), namely, the ability to link crime scenes in an investigative manner. We explore the potential for this tool to be used for intelligence purposes in the next section.

The main focus of our study was to investigate the potential contamination that could occur from sample to sample under two proposed mechanisms. Inspection of the comparisons that would apply to these mechanisms in Figures 3 and 4, do not show any instances of support for contamination having occurred, and have a similar distribution of *LR*s to the samples that are not paired by the proposed mechanisms. From a QA standpoint this is a desirable outcome that further demonstrates the high functioning of the existing QA processes in place at Forensic Science SA. From an investigative study standpoint, the results (while perhaps not exciting), demonstrate the functionality of mixture to mixture comparisons within a QA process.

5.2 The potential for intelligence gathering

As mentioned in section 5.1, the finding of two high *LR* values for samples within extraction batch 5 highlighted the ability for mixture to mixture comparisons to be used as an investigative

tool. To further demonstrate this functionality, we compared all mixture within this study (and additional samples not included in this study, but selected under the same criteria) so that a total of 340 samples were cross compared. This resulted in approximately 57,000 comparisons and approximately 329,000 comparisons of components within each mixture to components in other mixtures.

Figure 5 shows the results of the comparisons in the same format as Figures 3 and 4. Note that no cells have been bordered in this analysis as it was not the purpose of the analysis to look for contamination. Within Figure 5 there appear to be several instances of *LR*s > 1 million. To make this easier to view all rows and columns in Figure 5 that did not contain an *LR* > 1 million were removed, leaving the plots shown in Figure 6. The axis labels in Figure 6 represent the positions that these columns and rows held within Figure 5, i.e. where the bottom-left corner of the plot shown in Figure 5 (the origin) is row 1, column 1 and the top right corner is row 340, column 340 (the number of samples). Figure 6 shows 12 potential investigative leads that link crime scenes together. These could be passed on to police to further their investigations. This demonstration of using mixture to mixture comparison as an investigative tool has only been carried out on a very limited number of samples in comparison to the many thousands of mixed DNA profiles produced at Forensic Science SA each year. Putting these results into a wider perspective of all data produced within a forensic laboratory, it shows the enormous amount of investigative information that potentially lies dormant awaiting analysis.

Implementing mixture to mixture comparisons within a laboratory that already produces probabilistic mixture deconvolutions (i.e. the results of deconvolution using a probabilistic genotyping system, also called weights) only requires software to implement the mathematics described by Slooten [6]. Typically, jurisdictions will already have legislation in place that allows crime scene to crime scene comparisons, however the results of mixture to mixture comparison may require some small additional explanation to stakeholders (the main difference to a typical crime scene to crime scene match being that there is no actual interpreted single source profile that has been uploaded to a database). For those nations that already have the capability to search mixed DNA profiles against the database (as per [4]), they must already have the ability to load genotype probability distribution files (such as New Zealand) and so the extension to mixture to mixture comparison may be only a slight addition to the existing algorithms. For nations that are only able to match single source profiles against each other (such as Australia) the modifications required to national systems will be greater, and we suggest a more impactful first step would be to implement the capability to match mixture deconvolutions against single source profiles.

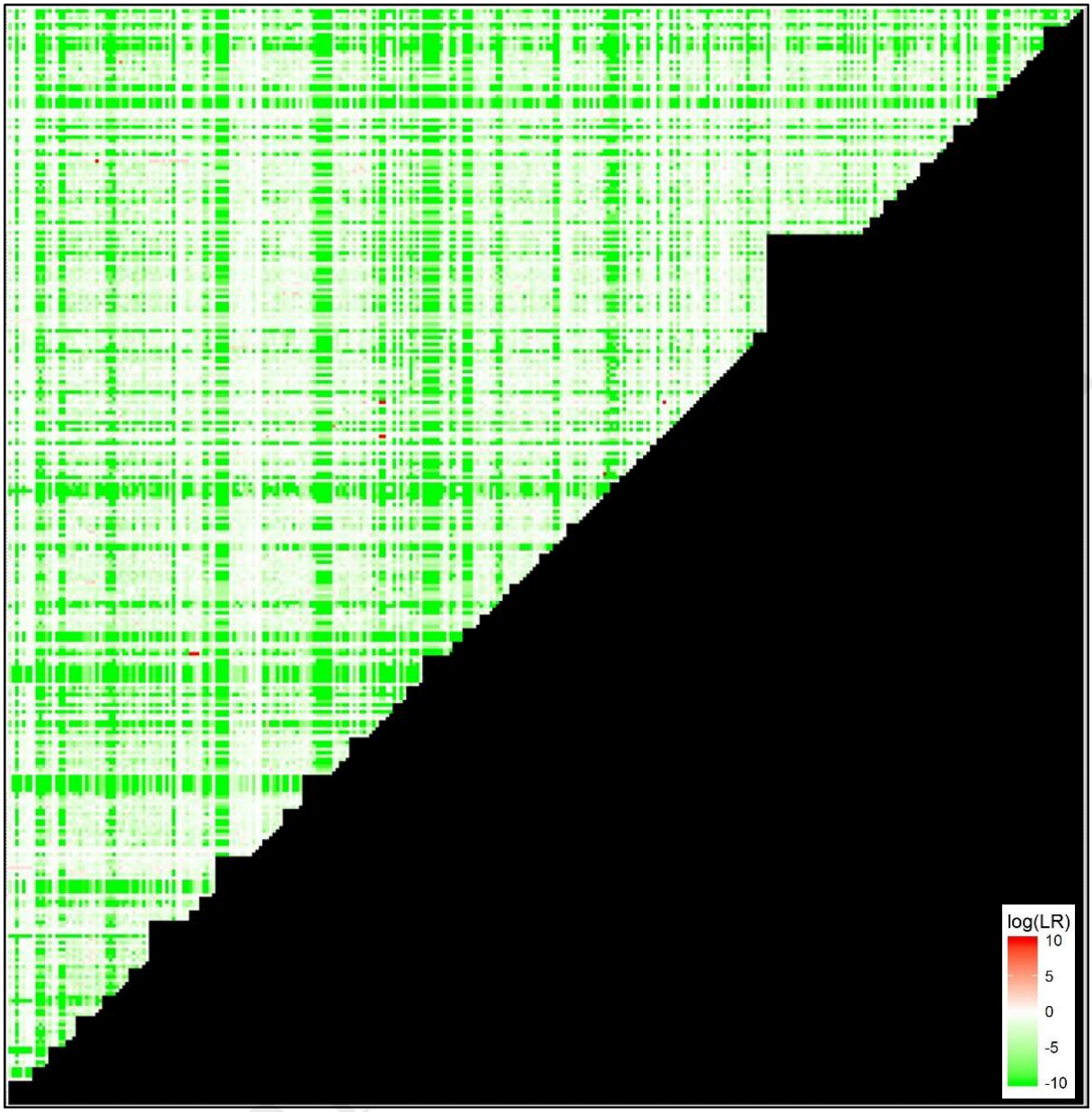


Figure 5: Log_{10}(LR) for sample to sample comparisons for samples within our study. Cells represent the comparison between samples listed in the column and row of the table. Log_{10}(LR) values are represented by colour (as per legend).

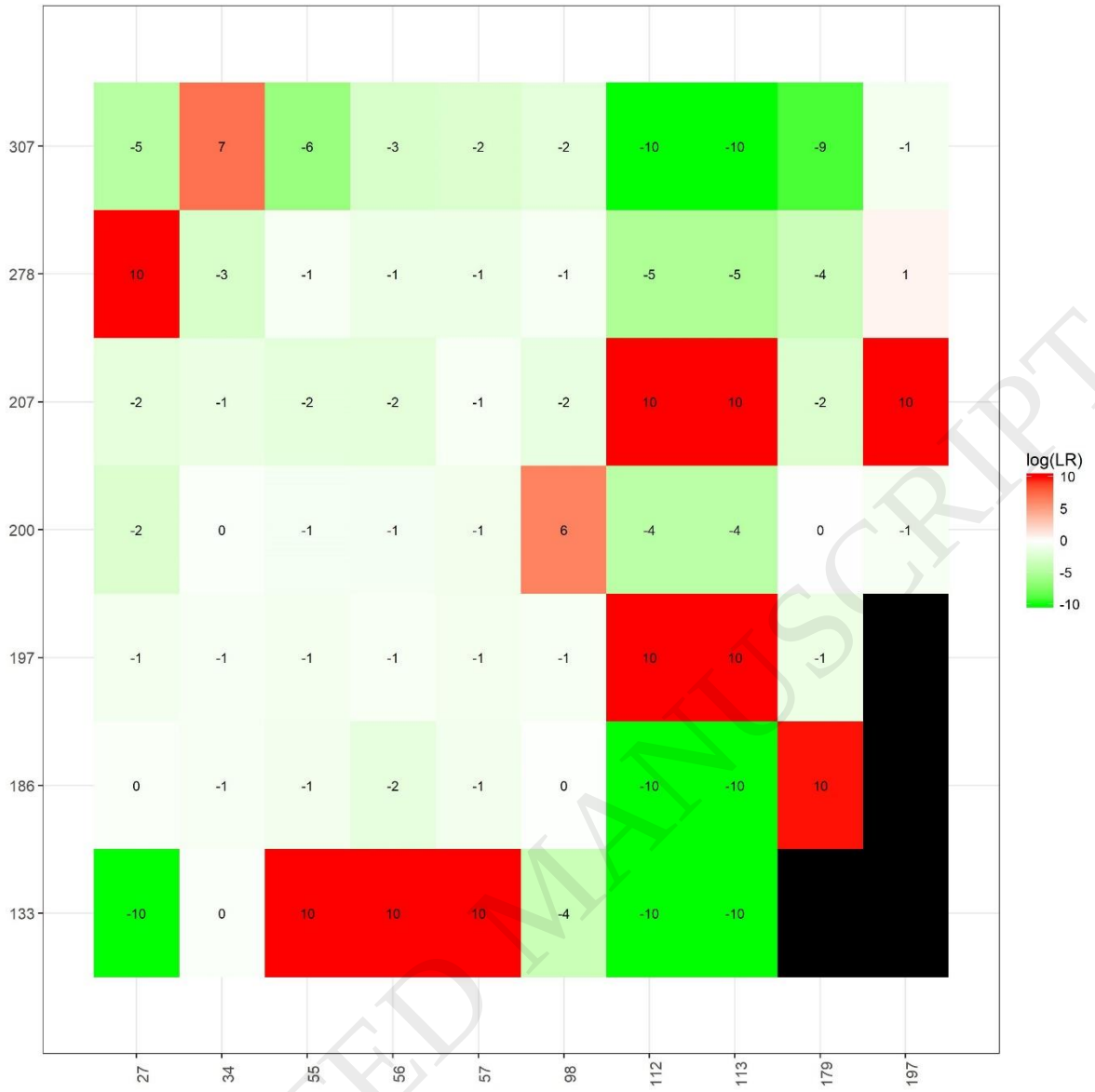


Figure 6: $Log_{10}(LR)$ for sample to sample comparisons for samples shown in Figure 5, but only for comparison that yielded an LR > 1 million. Cells represent the comparison between samples listed in the column and row of the table. $Log_{10}(LR)$ values are represented by colour (as per legend) and by numerical value in each cell. The axis values represent the position of the column or row in Figure 5, which ranges from (0,0) in the bottom-left to (340,340) in the top-right.

6.0: CONCLUSION:

In this work we have used the mathematics published by Slooten [6], which can compare mixed DNA profiles and calculate an *LR*, considering propositions:

- The DNA profiles have a common contributor
- The DNA profiles do not have any common contributors.

We apply this tool to the quality assurance realm, where we have used to it investigate the potential for sample to sample contamination to have occurred at either the DNA extraction or the electrophoresis stage. Our investigations found that there was no evidence of contamination caused by either potential mechanism within the data that we examined.

The first proposition given above (and the mathematics published by Slooten) specify a single common contributor. Being evidence samples, we are unsure whether there may in fact be more than one contributor in common and hence, technically, neither proposition need be true. Work by Bright et al. [10], showed that the behaviour of the *LR* when there were multiple contributors in common, was very similar to when there was only one contributor in common, and the *LR* obtained using the method described would approximate an *LR* considering the propositions:

- The DNA profiles have at least one common contributor
- The DNA profiles do not have any common contributors.

which is how we intuitively interpret the results in our work.

While no instances of contamination were found, the analyses did reveal a potential investigative link between cases. This highlighted the power of a mixture to mixture comparison tool to yield new investigative leads, when the case has not yielded an interpretable single source profile (and searching of any mixtures against a database of single source profiles, as described in [4], has not identified a potential contributor). To further demonstrate the investigative power of this tool we carried out a small 'database search' between all combinations of profiles used in our study. Even from this relatively small dataset a number of potential links were identified. As the number of profiles being compared within the pool increases the number of inter-case comparisons rises rapidly. We can only speculate as to how many case to case links would be revealed if this process were carried out on the hundreds of thousands of mixed DNA profiles sitting in laboratory archives.

Acknowledgements

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Table 1: Summary of extraction batches used in analysis

Extraction batch	Tip re-use	Samples in batch with deconvolutions	Number of contributors *N* = 1,2,3,4,5	Sample to sample comparisons (excluding intra-case)
EB 1	Yes	23	1,9,8,5,0	232
EB 2	Yes	4	3,1,0,0,0	5
EB 3	Yes	14	5,3,4,2,0	82
EB 4	Yes	24	5,9,6,3,1	249
EB 5	No	26	6,6,8,6,0	316
EB 6	No	28	10,7,4,7,0	333
EB 7	No	17	4,5,2,5,1	129
EB 8	No	26	2,12,9,3,0	297

Table 2: Summary of run batches used in analysis

Run batch	Samples in batch with deconvolutions	Number of contributors *N* = 1,2,3,4,5	Sample to sample comparisons (excluding intra-case)
RB 1	33	3,17,9,3,1	397
RB 2	23	0,9,9,5,0	219
RB 3	20	1,6,10,3,0	158
RB 4	24	8,11,4,1,0	237